PURIFICATION AND CHARACTERIZATION OF *C. BOTULINUM* TOXINS

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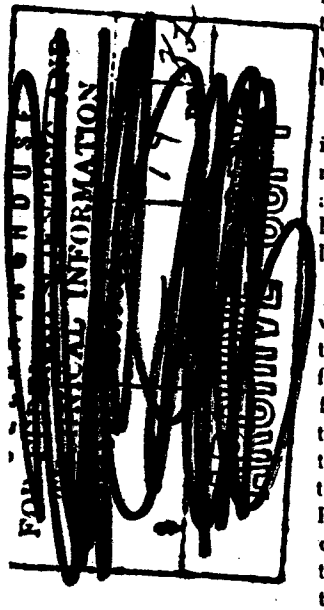
Five of the six types of *Clostridium botulinum* toxin that are now recognized have been obtained in a more or less purified form: They are types A, B, C, D, E. The recently discovered type F has not been purified. These toxins are termed "exotoxins," but are liberated into the culture medium upon autolysis after the culture has grown and not from the intact cell as the term "exotoxin" would imply. The formation of the toxins is not understood, but it is probable that they are formed in combination with the cell proteins and are liberated by enzymes. Another possibility is that they are formed in connection with a phage and subsequently are liberated by enzymes. Whatever mechanism is involved, type E is an exception in that its full toxicity becomes available only after treatment with trypsin. This fact was originally pointed out by Duff, Wright, and Yarinsky[1] at Fort Detrick. Bonventre and Kempe[2] observed that all types are activated by enzyme action and, with the exception of type E, these enzymes become available as the cells autolyze.

Many attempts have been made to purify the toxins. Snipe and Sommer[3] concentrated the toxin by isoelectric precipitation with acid at pH 3.5. While working at Fort Detrick, Lamanna and coworkers[4] obtained type A in crystalline form. Later type B was purified by Lamanna and Glassman,[5] but not in crystalline form. Purification of type C by extraction of the toxin from washed cells was reported by Boroff et al.[6] and by Katitch.[7] Since 1956, types A, B, C, D, and E have been obtained in a highly purified form at Fort Detrick by Dr. George Wright and his coworkers for the purpose of preparing high quality toxoids. Type E was also isolated by Dolman and his coworkers at the University of British Columbia.[8]



The isolation of these toxins has involved a variety of techniques including isoelectric precipitation with acid, shaking with chloroform, precipitation with ethanol, high-speed centrifugation, precipitation and crystallization from salt solutions and extraction with salt solutions, and, recently, chromatography on modified cellulose. The main steps in the purification procedure of each type are reviewed briefly below.

Lamanna was successful in isolating type A in crystalline form by precipitation with acid, shaking with chloroform, and crystallization from ammonium sulfate solution. Concurrently Abrams, Keegles, and Hottle[9] isolated the toxin in crystalline form but precipitated the toxin with sodium sulfate instead of shaking with chloroform. Duff et al.[10] improved the method of preparation and employed acid precipitation at pH 3.5, extraction of the toxin in 0.075 M calcium chloride, acid precipitation at pH 3.7, precipitation with 15 percent ethanol at -5°C, and finally crystallization from 0.9 M ammonium sulfate solution. The original purification of the type B toxin by Glassman and Lamanna involved acid precipitation at pH 3.5, extraction of the toxin at pH 2, and reprecipitation of the toxin with acid at pH 4 and a second time at pH 5. Duff et al.[11] employed practically the same procedure they used for the purification of type A except that they did not crystallize the type B from ammonium sulfate solution. Cardella et al.[12] purified type C by precipitation of the toxin from the culture in 25 percent ethanol at -5°C, extraction of the toxin from this precipitate with 0.05 M calcium chloride at pH 5.0, and reprecipitation of the toxin with 15 percent ethanol at -5°C. Cardella et al.[13] also purified type D by a

procedure very similar to that for the type C. The procedure involved precipitation of the toxin from the culture with 25 percent ethanol at -5°C, extraction with 0.075 M calcium chloride at pH 6.5, and reprecipitation of the toxin with 10 percent ethanol at -5°C. Another precipitation with ethanol at 10 percent was used for the type D. Fiock, Yarinsky, and Duff,[14] and Gordon et al.[15] isolated a highly purified toxin from type E cultures after treating the culture with 0.1 percent trypsin (pH 6.0) for 2 hours at 37°C. Their procedure involved precipitation of the toxin from the culture with ammonium sulfate at 60 percent saturation, extraction of the toxin from this precipitate with 0.075 M calcium chloride, pH 6.0, and precipitation of the toxin with ethanol at 25 percent at -5°C. Gerwing, Dolman, and Arnott,[16] and Gerwing and Dolman and coworkers[17] obtained highly purified type E toxin by precipitation of the toxin from the culture with ammonium sulfate and chromatography of the toxin on DEAE cellulose. Attempts in our laboratory to purify type A toxin by chromatography on anion-exchange resins were not successful however.

Apparently the chemical and physical properties of the different types vary enough so that no one universal procedure can be used for all types. The details of the chemical and physical properties are discussed subsequently.

ASSAY PROCEDURES

When work is carried out on the purification and characterization of the toxins, reliable assay methods are essential. In general, we use the quantal mouse assay; a mouse unit (MU), or LD_{50}, is defined as the amount of toxin that will kill 50 percent of a group of mice within 96 hours. Serial dilutions of a toxin solution are made in distilled water or in a gelatin-phosphate buffer (0.1 percent gelatin in 0.05 M sodium phosphate at pH 6.8) and adjusted so that the challenge dose is contained in 0.5 milliliter. White mice weighing 16 to 20 grams are injected intraperitoneally with 0.5 milliliter of the solution to be assayed. Eight or ten mice are usually used in each group and deaths are recorded for 96 hours. The percent kill is plotted against the dose on probit-log dose paper with the probit for 100 percent kill taken as the probit for 5 out of 6 killed plus ½ probit unit, and the probit for 0 percent kill taken as the probit of 1 out of 6 killed minus ½ probit unit.[18] The best straight line is fitted by inspection and the dose corresponding to probit 50 percent is read off the graph. A plot of this type is shown in Figure 1. A precision of ±25 percent is attainable with 10 mice on each dilution and sufficient dilutions are used (at least three) on each side of the 50 percent point to define the course of the line. For assaying cultures of partially purified preparations or food materials in our laboratory, dilution with either buffer or water has been found satisfactory, but for assaying highly purified preparations of the toxin, it is recommended that the above gelatin-phosphate buffer be used.

A much more rapid method of assay was studied at Fort Detrick (DeArmon et al.,[19] Lamanna et al.[20]) in which the time from challenge to death was taken as a measure of the toxin concentration. The assay is applicable to solutions containing between 100 and 100,000 LD_{50} in 0.5 milliliter. In this assay 50 mice (for a ±40 percent error) are injected intraperitoneally with 0.5 milliliter of the solution to be tested and the time from challenge to death is observed at 5- to 10-minute intervals. The relationship between time to death and dose is illustrated in Figure 2. The values for the MU in this figure are based on the quantal assay. This assay has the important advantage of obtaining an answer within about 1 to 4 hours, whereas the quantal (all or none) assay requires 96 hours. In the quantal assay most of the mice die in the first 48 hours. The disadvantage of the time-to-death assay is that

the animals must be observed continually over the time period, whereas daily observation is sufficient for the quantal assay. The precision of the quantal assay is greater particularly when a low percentage error is desired. If large errors (±40 percent) can be tolerated, both methods give about the same precision for an equal number of animals. Table 1, presented as a guide only, shows the calculated number of animals necessary for a certain precision for the two methods. It is based on data obtained on several hundred animals.[19] It should be pointed out that a linear response can be accepted only within certain parameters and even within these parameters the response only approximates linearity.

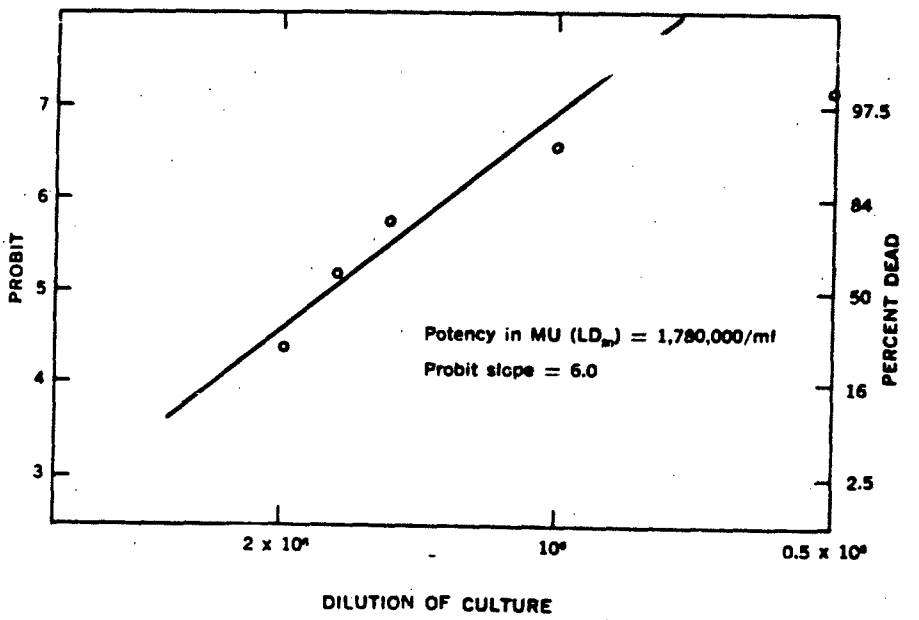


Figure 1 — Typical quantal (all-or-none) response of mice to dilutions of a culture of type A botulinum toxin. From preliminary trials the culture was estimated to contain about 2 x 10^6 MU per milliliter and dilutions were made accordingly. In this particular experiment 79 of 80 mice died at 0.5 x 10^6 dilution, 85 of 90 at 10^6, 43 of 55 at 1.5 x 10^6, 26 of 45 at 1.75 x 10^6, and 23 of 80 at 2 x 10^6.

On several occasions the question of a standard method of assay has been raised. Boor, Tresselt, and Schantz[21] have shown that many factors affect the results of assay. A well-defined medium and procedure for assay would be helpful in obtaining consistent results and in comparing results between laboratories, but the variability of animals from different sources would necessitate the use of a reference standard toxin solution to make the comparisons valid. A similar situation was encountered in the results of mouse assays for the paralytic poison in shellfish products. When a chemically defined pure poison was used as a standard, satisfactory results between laboratories were obtained.[22] At present, it should be possible to use the type A crystallized botulinum toxin as a standard for comparison. The purification and crystallization of many batches of the toxin in our laboratory have shown that the toxicity, ultra-violet absorption, sedimentation, and other properties define the purified protein sufficiently for its use as a standard. Type A toxin crystallized two times possesses 2.4 × 10^8 ±20 percent LD_{50} mouse units per milligram of nitrogen, has an absorbance of 10.67 per milligram of nitrogen in a 1-centimeter cell at 278

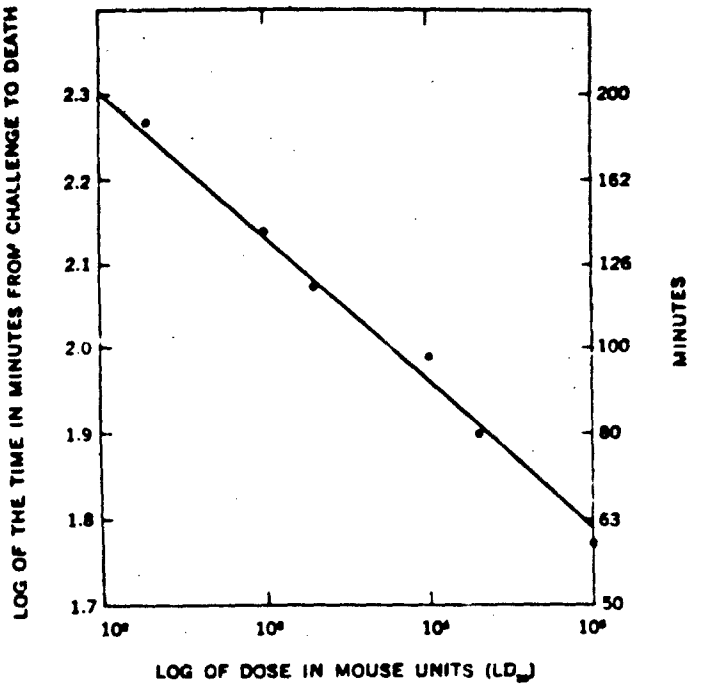


Figure 2 — Relationship between the dose of botulinum toxin and the time from challenge to death (dose given by intraperitoneal injection). The dose in each case was based on the quantal assay.

Table 1 — Precision of the assay method in terms of number of animals per assay.

Expected variability on log dose (95% confidence)	Converted to percentage error	Assay method	
		Graded	Quantal
		Mice per assay	Mice per assay[a]
±.025	±6	1800	369
±.050	±12	460	115
±.100	±26	114	52
±.150	±42	51	40
±.200	±59	30	36
Time to complete assays		~ 4 hr	96 hr

[a]The theoretical number includes 30 mice for range finding. The number used in assay yield a response in the range 16 to 84 percent to gain estimated variability.

millimicrons, has a ratio of absorption at 260 millimicrons to that at 278 millimicrons of 0.5 to 0.52, and is a single sedimenting component in the ultracentrifuge with a sedimentation constant of 17.3 S. Although the physical properties are in no way a measure of the toxicity, they define the purity of the protein upon which the toxicity is based.

When working with purified toxin in our laboratory, we have used the absorption data to measure the toxin concentration of a solution. The absorbance at 278

millimicrons in a cell with a light path of 1 centimeter divided by the factor 1.7 gives the concentration in milligrams of type A toxin per milliliter.

The crystalline toxin dissolved in acetate buffer at pH 3.8 has maintained its toxicity for a year or more. We recently found that storage of the toxin in buffers containing divalent anions, such as oxalate, imparts much greater stability to the crystalline toxin in solution.[23]

Another method of assay described by Cartwright and Lauffer[24] uses gold fish and is a quantal assay with a holding period of 36 hours.

A difficulty we have encountered on occasions in the assay of the toxin is an unexplained potentiation to the extent of 100- to 1,000-fold. This potentiation has been observed by others and is discussed by Boor, Tresselt, and Schantz.[21] Various proteinaceous substances such as serum, meat broth, and peptones have been reported to be the cause. Our experience indicated that the gelatin used in the buffers may have caused the potentiation, but if it was the cause, the conditions under which it acted are not known. All experiments in our laboratory to test the potentiation of various substances failed to show any marked effect. Gastric mucin showed a potentiation of threefold, but certain buffer salts in the absence of gelatin lowered the assay value to one-tenth that obtained in a gelatin phosphate buffer. When one experiences a potentiation by the addition of a particular substance to the medium used for assay, it is difficult to determine whether the value before or after potentiation should be taken as the correct one. Wentzel, Sterne, and Polson[25] experienced a marked potentiation (5,000 times) with the type D toxin when using a gelatin-phosphate buffer, and suggested that the buffer may cause a dissociation of the toxin and thus bring about an increase in specific toxicity. I will point out subsequently that the toxin can be dissociated into small units, but there is no evidence that the specific toxicity is increased more than twofold or threefold at the most.

CHARACTERIZATION

Some properties of the purified types of botulinum toxin are presented in Table 2. (See also a review by Lamanna.[26]) Considerable variation exists in their molecular weights and specific toxicities. Besides the differences in their serological behavior, the isolated proteins differ in their chemical and physical behavior. As far as we know all of the purified types are simple proteins and possess hemagglutinating properties, which I will discuss later. Some European workers have indicated that type E toxin is a lipoprotein.

It is difficult to determine the purity of a natural product of this type but certain criteria have been used as guidelines. A preparation is considered highly purified when it acts like a single substance in the toxin-antitoxin reaction, in the ultracentrifuge, and in electrophoresis. Since the toxin has a strong absorption at 278 millimicrons and contains no nucleic acids, we have used the ratio of the absorption at 260 millimicrons to that at 278 millimicrons as an index of purity. The lowest value attained thus far for the type A is 0.5. Experience has shown that preparations of type A that give this ratio are of equal purity.

Most of the studies on the chemical and physical properties of the toxins have been carried out on the type A because it was the first one available in purified form and in sufficient quantity for these studies. The crystals obtained by Lamanna, McElroy, and Eklund[27] and by Abrams, Kegeles, and Hottle[9] were white needles about 100 microns in length. Kegeles,[28] and Putnam, Lamanna, and Sharp[29, 30] studied some

of the physical properties of the type A toxin and found the diffusion coefficient ($D^{\circ}_{20^{\circ},w}$) to be about 2 x 10^{-7} cm^2 sec^{-1} and the sedimentation coefficient ($s^{\circ}_{20^{\circ},w}$) to be 17.3 S. On the basis of a partial specific volume of 0.75, the molecular weight calculated from S_{20} and D_{20} was 900,000. The isoelectric point was found at pH 5.6.

Table 2 — Comparison of some properties of the purified preparations of botulinum toxin.

Type	Molecular weight	Specific toxicity LD_{50} per gram[a]	Estimated purity of preparation	Form
A	900,000[b]	3.8×10^{10}	>98	Crystalline
B	Similar[c] to A	3.8×10^{10}	>98	Amorphorous
C	–	0.7×10^{10}	?	Amorphorous
D	Similar[d] to A	7.5×10^{10}	ca 90	Amorphorous
E	19,000[e]	0.6×10^{10}	>90	Amorphorous
F	–	–	–	–

[a]Calculated for the toxin on the basis that each type contains 16 percent nitrogen (LD_{50}/mg N x 160)
[b]Reference 30.
[c]References 11 and 5. Lamanna and Glassman isolated type B by another method and from sedimentation data suggested the molecular weight should be about 60,000.
[d]Reference 13.
[e]Reference 17.

Buehler, Schantz, and Lamanna[31] studied the amino acid composition of the toxin and found that it is composed of amino acids only and therefore is a simple protein. They found the toxin contained 16.2 percent nitrogen in contrast to 14.2 percent reported by Abrams. This difference is believed to be due to incomplete digestion of the toxin by the Kjeldahl procedure when a copper catalyst is used as recommended for other proteins. Use of a mercury catalyst and completion of the oxidation with a peroxide yielded the higher value of 16.2. This would indicate that the toxin possesses a structure that is more difficult to oxidize completely than that possessed by proteins in general. The analyses for the amino acids were performed by microbiological assay, and since the organisms used were only the natural isomers of amino acids, it is assumed that the toxin is composed of L-amino acids. The configuration of tryptophan and aspartic acid could not be ascertained, however, because of the methods used for hydrolyses. Table 3 shows the apparent number of moles of amino acid residues in a mole toxin having a molecular weight of 900,000. The total nitrogen content of 16.2 percent was completely accounted for in the 19 acids listed. Also the total sulfur of 0.436 percent was completely accounted for in the cysteine, half-cystine, and methionine content. Cysteine is the limiting amino acid and a calculation of the molecular weight on this basis gave a value of 45,000 or 1/20th of the molecular weight of 900,000.

Further studies of the amino acid analysis of the toxin have been made by Stefanye et al.[23] using the chromatographic techniques of Spackman, Stein, and Moore.[32] The results of these analyses are in general agreement for 14 of the amino

acids, but some change may be made in the values for tyrosine, phenylalinine, tryptophane, serine, and glycine.

Table 3 — Apparent number of moles of amino acid residues per mole of *Clostridium botulinum* type A toxin.[a]
(Mol Wt = 900,000)

Lysine	477	Glutamic acid	953
Histidine	60	Asparagine	1,370
Arginine	239	Serine	374
Tyrosine	672	Threonine	642
Phenylalanine	64	Cysteine	20
Tryptophan	82	Half-cystine	40
Valine	406	Methionine	64
Leucine	708	Proline	203
Isoleucine	820	Glycine	166
		Alanine	394
Total residues			7,754

[a]Reference 31.

Attempts to explain the high toxicity of the molecule on the basis of its chemical and physical properties or its chemical composition have not been fruitful. No prosthetic group was found that could be disassociated from the protein, although one composed of amino acids might exist and this possibility should not be overlooked in future studies.

Boroff and Fitzgerald[33] and Boroff[34] found that in many cases where the toxicity of the molecule was destroyed by treatment the fluorescence was also lost. Their interpretation of this phenomenon was that toxicity and fluorescence were invested in a common structure and fluorescence was a measure of toxicity. Schantz, Stefanye, and Spero[35] found that treatments that destroy toxicity usually destroy fluorescence, but in 6 M urea the toxin is destroyed without loss of fluorescence. These data indicate that toxicity and fluorescence depend upon different bonds or structures and that fluorescence is not a direct measure of toxicity.

Distortion of the molecule by spreading on a large surface[36] or treatment with 6 M urea at room temperature[35] causes a complete loss of toxicity. Dialysis of the urea from the protein does not bring about a return of any of the toxicity. Weil et al.[37] found that toxicity is destroyed by photo-oxidation in the presence of methylene blue.

Schantz and Spero[38] studied the reaction of the toxin with ketene and found that it was readily detoxified with this reagent. When 5 percent of the free amino groups had reacted with the ketene, 43 percent of the toxicity was lost, and when 19 percent of the amino groups had reacted, 98 percent of the toxicity had disappeared. Reaction of the ketene with the phenolic hydroxy and free sulfhydryl groups did not appear to affect the toxicity. Spero and Schantz[39] found that deamination of the toxin with nitrous acid caused a rapid detoxification, which was interpreted as being due to deamination. Spero[40] found, on the basis of a thermodynamical considera-

tion, that the decrease in toxicity due to an increase in pH is associated with the ionization of a small number of the ε-amino groups of lysine. The accumulation of evidence thus far definitely indicates that the toxicity is a result of a particular conformation (coiling and folding) of the molecule, in which the free amino groups, at least, are involved.

Studies of the stability of the toxin showed that it is readily denatured by heat, strong alkali, and many oxidizing agents. Cartwright and Lauffer[41] found that the toxin in solution at pH 6.9 could be heated at 40°C for 1 hour without loss of toxicity, but at 50°C or above a rapid detoxification took place within minutes. These data are illustrated in Figure 3. The thermal inactivation curves presented by these investigators indicate that the toxin splits into two components, each having its own rate of inactivation. Spero[40] found that the toxin dissolved in buffered solutions at a temperature of 15°C and up to pH 10.58 were very stable for at least 3 hours, but 50 percent of the toxicity was lost at pH 10.79 in 15 minutes, and 90 percent or more of the toxicity was lost at pH 11.2 in 1 to 2 minutes. These data are illustrated in Figure 4.

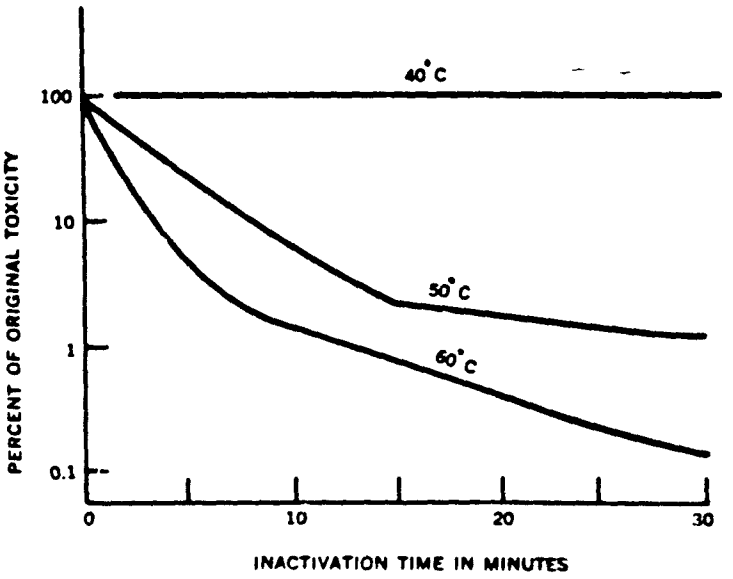


Figure 3 — Thermal inactivation curves for botulinum toxin, type A, at several temperatures. Reference 41. (Courtesy of Society for Experimental Biology and Medicine.)

An important question often raised regarding the type A toxin is the possibility of this large molecule (mol wt 900,000) dissociating into smaller units. It is difficult to understand how a molecule of this size could pass through the intestinal wall and through the various body membranes to the site of action. Wagman and Bateman[42, 43] and Wagman[44] were the first to demonstrate in the ultracentrifuge that the molecule under the proper conditions of pH and ionic strength dissociates into small toxic units. Toxin dissolved in buffers in the pH range 6.5 to 8.0 and ionic strength 0.13 and above gradually dissociates into slowly sedimenting units with molecular weights from 40,000 to 100,000.

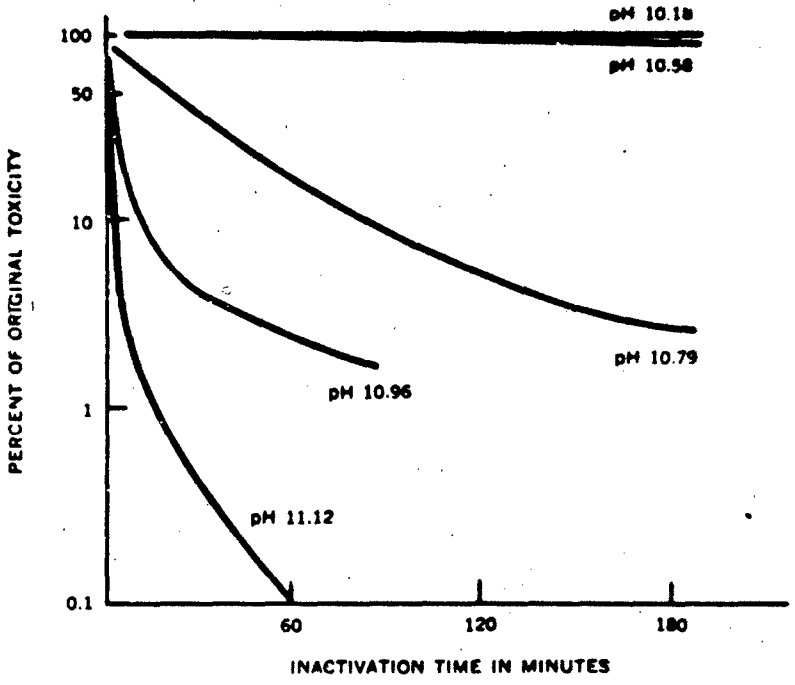


Figure 4 — The inactivation of botulinum toxin, type A, at 15°C at several pH values. Reference 40.

Soon after Lamanna obtained the type A in crystalline form and it was found to be homogenous, he also discovered that it possessed hemagglutinating properties[45] and that these properties could be retained without retention of toxicity.[46, 47] The molecule therefore performs two functions that are apparently due to different structures in the molecule. Since the molecule dissociates into small toxic units under the proper conditions, the question is raised as to whether the small units are identical toxic pieces that polymerize to form a large unit with hemagglutinating properties or whether some of the pieces possess toxic properties and others possess hemagglutinating properties. Sufficient data to answer these questions definitely are not available at this time. In our laboratories we have attempted to measure the diffusion rate of toxicity and hemagglutination in agar gel and have found that the toxicity moves much faster than the hemagglutinating properties. In these experiments the concentration was 0.05 millogram per milliliter in 0.1 M phosphate buffer at pH 6.8. The estimated molecular weight of the toxin based on the diffusion rate was between 10,000 and 20,000.[48] Heckley, Hildebrand, and Lamanna[49] studied the sedimentation rate of the toxic activity in lymph of orally poisoned rats and concluded that the size of the molecule in the lymph must be much smaller than that fed to the animals. Riesen, Sumyk, and Hawrylewicz[50] have shown that the toxin dissociates to some extent, at least, into small units when treated for 4 hours in 0.1 N hydrochloric acid and that these small units can be separated from the remaining undissociated toxin under the proper conditions on Sephadex columns. Recently Wagman[51] obtained a toxic dialyzable unit that has a molecular weight of 3,800 from a pepsin digest of toxin previously treated in alkali at pH about 9. The yields of the

small toxic unit were very low, but the studies indicate a possible means of liberating the toxic entity of the molecule. The existence of a toxic unit of this small size would mean that all units that might be cleaved from the large toxin molecule could not be representative of the parent molecule because of the limiting amount of cysteine residues. This means that the characterization and structure determination of the toxic entity must await the purification of these small dissociated or cleaved units in sufficient quantity for these studies. In our laboratories at Fort Detrick we have prepared many grams of the crystalline toxin with the hope of accomplishing this interesting task.

REFERENCES

1. Duff, J. T., G. G. Wright, and A. Yarinsky. Activation of *Cl. botulinum* type E toxin by trypsin. J. Bacteriol. 72:455-60. 1956.

2. Bonventre, P. F., and I.. L. Kempe. Physiology of toxin production by *Clostridium botulinum* types A and B. III. Effect of pH and temperature during incubation on growth, autolysis, and toxin production. Appl. Microbiol. 7:374-77. 1959.

3. Snipe, P. T., and H. Sommer. Studies on botulinus toxin. 3. Acid precipitation of botulinus toxin. J. Infectious Diseases. 43:152-60. 1928.

4. Lamanna, C., H. W. Eklund, and O. E. McElroy. Botulinum toxin (type A); including a study of shaking with chloroform as a step in the isolation procedure. J. Bacteriol. 52:1-13. 1946.

5. Lamanna, C., and H. N. Glassman. The isolation of type B botulinum toxin. J. Bacteriol. 54:575-84. 1947.

6. Boroff, D. A., M. Raynaud, and A. R. Prevot. Studies of toxin of *Clostridium botulinum* type C. J. Immunol. 68:503-11. 1952.

7. Katitch, R. V. Sur une technique d'extraction de la toxine *Clostridium botulinum* (C) a portir des corps microbiens. Rev. immunol. 16:384-89. 1952.

8. Dolman, C. E. Personal communication. See Gerwing et al. 1964.

9. Abrams, A., G. Kegeles, and C. A. Hottle. The purification of toxin from *Clostridium botulinum* type A. J. Biol. Chem. 164:63-79. 1946.

10. Duff, J. E., G. G. Wright, J. Klerer, D. E. Moore, and R. H. Bibler. Studies on immunity to toxins of *Clostridium botulinum*. I. A simplified procedure for isolation of type A toxin. J. Bacteriol. 73:42-47. 1956.

11. Duff, J. T., J. Klerer, R. H. Bibler, D. E. Moore, C. Gottfried, and G. G. Wright. Studies on the immunity to toxins of *Clostridium botulinum*. II. Production and purification of type B toxin for toxoid. J. Bacteriol. 73:597-601. 1957.

12. Cardella, M. A., J. T. Duff, C. Gottfried, and J. S. Begel. Studies on immunity to toxins of *Clostridium botulinum*. IV. Production and purification of type C toxin for conversion to toxoid. J. Bacteriol. 75:360-65. 1958.

13. Cardella, M. A., J. A. Duff, B. H. Wingfield, and C. Gollfried. Studies on immunity to toxins of *Clostridium botulinum*. VI. Purification and detoxifica-

tion of type D toxin and the immunological response to toxoid. J. Bacteriol. 79:372-78. 1959.

14. Fiock, M. A., A. Yarinsky, and J. T. Duff. Studies on immunity to toxins of *Clostridium botulinum*. VII. Purification and detoxification of trypsin-activated type E toxin. J. Bacteriol. 82:66-71. 1961.

15. Gordon, M., M. A. Fiock, A. Yarinsky, and J. T. Duff. Studies on immunity to toxins of *Clostridium botulinum*. III. Preparation, purification, and detoxification of type E toxin. J. Bacteriol. 74:533-38. 1957.

16. Gerwing, J., C. E. Dolman, and D. A. Arnott. Purification and activation of *Clostridium botulinum* type E toxin. J. Bacteriol. 81:819-22. 1961.

17. Gerwing, J., C. E. Dolman, M. E. Reichmann, and H. S. Bains. Purification and molecular weight determination of *Clostridium botulinum* type E toxin (In press 1964).

18. Weiss, E. S. An abridged table of probits for use in the graphic solution of the dosage effect curve. Am. J. Public Health. 38:22-24. 1948.

19. DeArmon, I. A., E. J. Schantz, L. Spero, F. Klein, and R. E. Lincoln. Studies on the feasibility of a rapid bioassay for botulinum toxin. U. S. Public Health Service Symposium, Atlanta, Ga., May 1958.

20. Lamanna, C., W. I. Jensen, and I. Bross. Body weight as a factor in the response of mice to botulinal toxins. Am. J. Hyg. 62:21-28. 1955.

21. Boor, A. K., H. B. Tresselt, and E. J. Schantz. Effect of salts and colloids on potency of botulinum toxin. Proc. Soc. Exptl. Biol. Med. 89:270-72. 1955.

22. Schantz, E. J., E. F. McFarren, M. L. Schafer, and K. H. Lewis. Purified shellfish poison for bioassay standardization. J. Assoc. Offic. Agr. Chemists. 41:160-68. 1958.

23. Stefanye, D., R. T. Iwamasa, E. J. Schantz, and L. Spero. Effect of guanidinium salts on the toxicity of botulinum toxin. Biochim. et Biophys. Acta. In press. 1964.

24. Cartwright, T. E., and M. A. Lauffer. Assay of botulinum A toxin with goldfish. Proc. Soc. Exptl. Biol. Med. 81:508-11. 1952.

25. Wentzel, L. M., M. Sterne, and A. Polson. High toxicity of pure botulinum type D toxin. Nature. 166:739-40. 1950.

26. Lamanna, C. The most poisonous poison. Science. 130:763-72. 1959.

27. Lamanna, C., O. E. McElroy, and H. W. Eklund. The purification and crystallization of *Clostridium botulinum* type A toxin. Science. 103:613-14. 1946.

28. Kegeles, G. The molecular size and shape of botulinus toxin. J. Am. Chem. Soc. 68:1670. 1946.

29. Putnam, F. W., C. Lamanna, and D. G. Sharp. Molecular weight and homogeneity of crystalline botulinus A toxin. J. Biol. Chem. 165:735-36. 1946.

30. Putnam, F. W., C. Lamanna, and D. G. Sharp. Physiochemical properties of crystalline *Clostridium botulinum* type A toxin. J. Biol. Chem. 176:401-12. 1948.

31. Buehler, H. J., E. J. Schantz, and C. Lamanna. The elemental and amino acid composition of *Clostridium botulinum* type A toxin. J. Biol. Chem. 169:295-302. 1947.

32. Spackman, D. H., W. H. Stein, and S. Moore. Automatic recording apparatus for use in chromatography of amino acids. Anal. Chem. 30:1190. 1958.

33. Boroff, D. A., and J. E. Fitzgerald. Fluorescence of the toxin of *Clostridium botulinum* and its relation to toxicity. Nature. 181:751-52. 1958.

34. Boroff, D. A. Studies of the toxin of *Clostridium botulinum*. IV. Fluorescence of the toxin of *Clostridium botulinum* and its relation to toxicity. Intern. Arch. Allergy. 15:74-90. 1959.

35. Schantz, E. J., D. Stefanye, and L. Spero. Observations of the fluorescence and toxicity of botulinum toxin. J. Biol. Chem. 235:3489-91. 1961.

36. Schantz, E. J., and J. Bateman. Unpublished data. 1961.

37. Weil, L., T. S. Seibles, L. Spero, and E. J. Schantz. Photooxidation of crystalline *Clostridium botulinum* type A toxin in the presence of methylene blue. Arch. Biochem. Biophys. 68:308-13. 1957.

38. Schantz, E. J., and L. Spero. The reaction of botulinum toxin type A with ketene. J. Am. Chem. Soc. 79:1623-25. 1957.

39. Spero, L., and E. J. Schantz. The reaction of botulinum toxin type A with nitrous acid. J. Am. Chem. Soc. 79:1625-28. 1957.

40. Spero, L. The alkaline inactivation of botulinum toxin. Arch. Biochem. Biophys. 73:484-91. 1958.

41. Cartwright, T. E., and M. A. Lauffer. Temperature effects on botulinum A toxin. Proc. Soc. Exptl. Biol. Med. 98:327-30. 1958.

42. Wagman, J., and J. B. Bateman. The behavior of the botulinus toxins in the ultracentrifuge. Arch. Biochem. Biophys. 31:424-30. 1951.

43. Wagman, J., and J. B. Bateman. Botulinum type A toxin: Properties of a dissociated product. Arch. Biochem. Biophys. 45:373-83. 1953.

44. Wagman, J. Isolation and sedimentation study of low molecular weight forms of type A botulinus toxin. Arch. Biochem. Biophys. 50:104-12. 1954.

45. Lamanna, C. Hemagglutination by botulinal toxin. Proc. Soc. Exptl. Biol. Med. 69:332-36. 1948.

46. Lamanna, C., and J. P. Lowenthal. The lack of identity between hemagglutinin and the toxin of type A botulinal organism. J. Bacteriol. 61:751-52. 1951.

47. Lowenthal, J. P., and C. Lamanna. Factors affecting the botulinal hemagglutination reaction and the relationship between hemagglutinating activity and toxicity of toxin preparations. Am. J. Hyg. 54:342-53. 1951.

48. Schantz, E. J., and M. A. Lauffer. Diffusion measurements in agar gel. Biochemistry. 1:658-63. 1962.

49. Heckley, R. J., G. J. Hildebrand, and C. Lamanna. On the size of the toxic

particle passing the intestinal barrier in botulism. J. Exptl. Med. 111:745-49. 1960.

50. Riesen, W. H., G. Sumyk, and E. J. Hawrylewicz. Dissociation of botulinum toxin with acid. To be presented at Intern. Congr. Biochemistry, New York, N.Y., July 26-31, 1964.

51. Wagman, J. Low molecular weight forms of type A botulinum toxin. II. Action of pepsin on intact and dissociated toxin. Arch. Biochem. Biophys. 100:414-21. 1963.

DISCUSSION FROM THE FLOOR

Dr. Schantz: Dr. Foster has asked about the European workers who published that the type E toxin is a lipoprotein. As far as we know from our work all types are simple proteins.

Dr. Dolman: I have no evidence of the lipoprotein hypothesis. I find it most interesting that the type A molecule, whose molecular weight approaches a million, was very difficult to reconcile with its absorption and with an observation made during the war by two Canadian workers at Kingston that crystalline type A toxin was not toxic for mice by mouth. There is a possibility that the toxin can be split into active components having molecular weights of the order of 20,000 to 70,000. This is much more physiologically acceptable and is to be correlated with our own unpublished findings that the type E molecule seems to have a molecular weight of only 19,000. There is no evidence in our work of a lipoprotein structure.

Dr. Lamanna: With type A toxin, a dose perhaps 50,000 times larger is required to kill mice by mouth than by IP injection.

Dr. D. A. Boroff: I wish to side with Dr. Lamanna with regard to oral toxicity. The crystalline toxin is toxic to mice. In my opinion, only 10 times the IP dose is necessary to kill the mouse by mouth.

Now, I should like to make a number of other comments about Dr. Schantz's splendid presentation. I was glad to hear that fluorescence is used for the estimation of the purity of the toxin in solution, and that the intravenous route is useful for a rapid estimation of toxicity. I should also like to comment about hydrogen bonds and their importance in toxicity. The present concept of combining sites is not that one amino acid or a number of amino acids strung side by side make up a combining site; it is that a group of amino acids may be in the same peptide chain or in the distal peptide chains, brought together by a hydrogen bond into a configuration that makes up a reactive site. If we break that bond and stretch the molecule of protein, we then destroy the special configuration of the molecule and, therefore, may destroy its activity.

We have recently completed a piece of work on the toxin Dr. Schantz so kindly gave us, regarding the role of tryptophan in making the molecule toxic. The crystalline toxin is the only preparation worthwhile working with, from the standpoint of purity, and we have tried to determine the significance of certain amino acids within the molecule of the toxin. Our attention was attracted to tryptophan because fluorescence depends on this amino acid. Whenever we destroyed fluorescence we destroyed the toxicity as well. Also, tryptophan is needed in relatively large amounts in the culture medium for toxin production. Another point is that

there is a peculiar property of serotonin that reduces the effect of the toxin when injected prior to the toxin. I do not want to go into the mechanism, but serotonin is, as you know, a derivative of tryptophan. All this evidence directed our attention to tryptophan as an active amino acid in the toxin. We attempted to destroy the tryptophan in the intact molecule; the only method we found was photo-oxidation, which Dr. Schantz has tried so successfully in cooperation with Dr. Weil. Only the tryptophan is destroyed by photo-oxidation if the pH is 3.5. We came to the conclusion that when we destroyed 26 moles of tryptophan out of about 80 in the molecule of 900,000 molecular weight, a considerable amount of toxicity was lost. When we destroyed about 56 moles of tryptophan, we lost 99 percent of the toxicity.

One more thing of interest is the stability of the toxin. It has been said that if you allow the toxin to warm up the toxicity is lost. My assistant once left the toxin on the table and forgot to put it in the icebox. To our great surprise, this sample showed a higher titer than the sample kept in the icebox. Now, we routinely warm up the toxin to room temperature before we test it.